ASTROBIOLOGY
Volume 4, Number 3, 2004

Research Paper

Evaluation of Various Cleaning Methods to Remove *Bacillus* Spores from Spacecraft Hardware Materials

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ABSTRACT

A detailed study was made of the biological cleaning effectiveness, defined in terms of the ability to remove bacterial spores, of a number of methods used to clean hardware surfaces. Aluminum (Al 6061) and titanium (Ti 6Al-4V) were chosen for the study as they were deemed the two materials most likely to be used in spacecraft extraterrestrial sampler construction. Metal coupons (1 cm × 2.5 cm) were precleaned and inoculated with 5.8×10^3 cultivable *Bacillus subtilis* spores, which are commonly found on spacecraft surfaces and in the assembly environments. The inoculated coupons were subsequently cleaned using: (1) 70% isopropyl alcohol wipe; (2) water wipe; (3) multiple-solvent flight-hardware cleaning procedures used at the Jet Propulsion Laboratory (JPL); (4) Johnson Space Center-developed ultrapure water rinse; and (5) a commercial, semi-aqueous, multiple-solvent (SAMS) cleaning process. The biological cleaning effectiveness was measured by agar plate assay, sterility test (growing in liquid media), and epifluorescent microscopy. None of the cleaning protocols tested completely removed viable spores from the surface of the aluminum. In contrast, titanium was capable of being cleaned to sterility by two methods, the JPL standard and the commercial SAMS cleaning process. Further investigation showed that the passivation step employed in the JPL standard method is an effective surface sterilant on both metals but not compatible with aluminum. It is recommended that titanium (Ti 6Al-4V) be considered superior to aluminum (Al 6061) for use in spacecraft sampling hardware, both for its potential to be cleaned to sterilization and for its ability to withstand the most effective cleaning protocols. Key Words: Spacecraft materials—Cleaning—Spores—Disinfection. Astrobiology 4, 377–390.

INTRODUCTION

THE SEARCH FOR LIFE on other planets (*in situ* or sample-return missions) will likely involve ultrasensitive detection of biosignatures. Biological contaminants on spacecraft outbound from Earth to other planetary bodies could contaminate those pristine environments and compromise both *in situ* life-detection experiments and returned extraterrestrial samples by yielding false positives. Protecting other solar system bodies from biological contamination originating from

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Earth is termed "forward contamination." Life detection missions require an unprecedented degree of control of the bioburden (organisms, their remnants, and organic matter in general) carried from Earth to other planets (Space Study Board, 1992). This is particularly true of a biosampler and its cache. Hence, the materials in contact with the sample to be collected should be chosen carefully for their ability to be cleaned. Towards that end, various cleaning methodologies must be evaluated for their abilities to remove microbial contamination. Our goal in this area of research is to identify and evaluate simple and effective combinations of cleaning methods and materials suitable for use on spacecraft hardware. This study focuses on two candidate materials common to the aerospace industry, which are likely to be considered for sample handling mechanisms: aluminum and titanium.

Over a period of years, the medical industry has developed a variety of disinfection methods and materials to remove biological contaminants from medical instruments (Penna *et al*., 2001). For example, quaternarium ammonium compounds are used in the disinfection of beds, matrix, and general hard surfaces to minimize cross-contamination. Solutions of chlorhexidine are widely used for cleaning contact lenses (0.005–0.006%). Glutaraldehyde and formaldehyde, which exhibit high bactericidal, sporicidal, fungicidal, and virucidal effectiveness, are widely used for high-level disinfection of critical and semi-critical medical facilities. Despite their antimicrobial effectiveness and beneficial annual cost, aldehydes are potentially carcinogenic, and their use requires a safe work environment and training program to ensure worker safety standards (McDonnell and Russell, 1998; Weber and Rutala, 1998). Immersion of non-critical and semi-critical items in ethanol at 70% overnight has replaced the use of phenol and formaldehyde solutions, and has been shown to be successful (Diaz *et al*., 2000).

Acids (peracetic acid) and buffers (sodium hypochlorite) are traditionally used as disinfectants but are corrosive to various metal surfaces. Although the 2% glutaraldehyde-based formulations, 6% stabilized hydrogen peroxide, and peracetic acid are among germicides categorized as chemical sterilants, they should be applied after adequate cleaning (Rutala and Weber, 1998; Weber and Rutala, 1998), following proper guidelines with regard to organic load, contact time, temperature, and pH. Hydrogen peroxide, a powerful oxidizing agent applied on non-critical items, is easily handled and non-toxic. It is a mainstay in metal surface treatment, and causes no damage to medical and dental devices in health care routine (Sagripanti and Bonifacino, 1997).

Spaceflight hardware currently undergoes routine gross cleaning to remove major contaminants, followed by precision cleaning as needed. Assembled flight-hardware surfaces are also routinely cleaned using isopropyl alcohol (IPA) wipes. However, some contaminants are resistant to standard cleaning techniques. While mapping microbial footprints of various spacecraft assembly facilities, Venkateswaran *et al*. (2001) and La Duc *et al*. (2003, 2004) showed that the majority of cultivable species recovered were spore-formers. Spores tend to exhibit adhesive characteristics that make them more difficult to remove (Hamon and Lazazzera, 2001; Faille *et al*., 2002). In addition, the *Bacillus* species commonly isolated from the spacecraft assembly facilities are resistant to various sterilization treatments such as desiccation and oxidizing and ionizing agents (Venkateswaran *et al*., 2001, 2003; La Duc *et al*., 2003; Link *et al*., 2004). We report here on the comparative effectiveness of various cleaning technologies currently in use in the aerospace and medical industries, which were developed to remove biological agents, especially bacterial spores, from spacecraft-qualified materials.

MATERIALS AND METHODS

Spacecraft-qualified materials

Two materials among those under consideration for use in the fabrication of *in situ* and sample-return hardware sample collection and processing systems were selected for study (aluminum Al 6061-T6 and titanium Ti 6Al-4V), based on the recommendation of the Jet Propulsion Laboratory (JPL) Spacecraft Assembly Engineering Group (personal communication). The mill-finished aluminum (15 rms) (in percent: 0.65 Si, 0.44 Fe, 0.27 Cu, 0.02 Mn, 0.96 Mg, 0.20 Cr, 0.02 Ti) and titanium sheets (32 rms) (in percent: 0.02 C, 0.152 O, 0.15 Fe, 0.015 N, 6.4 Al, 3.9 V) were procured from the JPL in-house machine shop and cut into 1×2.5-cm "coupons." Care was taken to avoid any scratches on the surface of the materials, which were visually inspected and re-

jected if they were scratched or did not have smooth edges.

Precleaning and sterilization

All coupons were precleaned with clean-room-grade polyester wipes (Coventry 6209 c-prime, Freon-washed; Tech Spray, L.P., Amarillo, TX) and then saturated with acetone to remove residual adhesive. This was followed by a Freon-vapor degreasing for 1 h, with subsequent rinsing with IPA and drying in filtered air at room temperature. The coupons were then placed in a single layer into glass petri dishes, covered in steam autoclave bags, and sterilized at 121°C for 15 min, followed by a 30-min drying cycle. Controls such as uncleaned and unsterilized coupons were also included in the study for comparative analysis.

Microbial seeding

Spores of *Bacillus subtilis* ATCC 6633 procured from Difco Laboratories (Detroit, MI) were used to inoculate the coupons. These are representative of spores commonly found on spacecraft surfaces and spacecraft assembly environments, and are some of the more difficult to remove. The washed spore suspension was diluted in sterile certified 18 MΩ water, from Sigma (St. Louis, MO). The concentration of initial inoculum was enumerated by microscopy for total number of spores (La Duc *et al.*, 2004) and by spread plate assay for total number of cultivable spores as described below. Each precleaned sterile coupon was seeded with 100 μl of the spore suspension and was calculated to contain 1.4×10^4 total spores, 5.8×10^3 of them cultivable. Some coupons were not inoculated with spores for comparative analysis.

Cleaning methods

In this study, five cleaning methodologies were evaluated to decontaminate *B. subtilis* spores. Unless otherwise indicated, all cleaning was performed in a Class 100, *i.e.*, the number of >0.5-μm particles is <100 per cubic foot, biohazard hood or a Class 100 laminar flow bench. All personnel wore powder-free nitrile gloves. Cleanliness was maintained as prescribed in the procedures of MIL-STD-1246C (Military Standard 1246C, 1998). Care was taken in handling, and high-grade chemicals were used to minimize contamination. In all five cleaning methodologies employed, six replicates were employed to achieve reproducibility as well as statistical significance.

The following cleaning methods were utilized and compared:

1. *NASA standard IPA cleaning*. Coupons were wiped with clean-room-grade polyester wipes saturated with 70% IPA solution.
2. *Ultrapure water (UPW) cleaning*. As previous studies have revealed that microbial cells are best removed with water (data not shown), coupons were wiped with clean-room-grade polyester wipes saturated with certified 18 MΩ water (Sigma).
3. *Multiple-solvent (JPL) cleaning*. This is the recommended method as described in JPL document FS505146C, General Cleaning of Materials, Rev. E (Jet Propulsion Laboratory, 1990). Briefly, coupons were treated by ultrasonic cleaning with acetone and IPA, followed by alkaline cleaning with Oakite 61B (Chemetall, Bletchley, UK). Subsequently, coupons were rinsed with deionized water and dried with clean, dry nitrogen. For titanium, there was an additional nitric acid passivation step involved after alkaline cleaning. The passivation consisted of a 30-min exposure to 5 *M* nitric acid at 24°C.
4. *Detergent precleaning and UPW cleaning*. A protocol for cleaning aluminum and stainless steel hardware was developed at Johnson Space Center (JSC) and used for cleaning the Genesis Mission flight hardware and interplanetary dust collectors. Briefly, very dilute (2 drops/L of warm UPW) Joy® detergent [Proctor and Gamble (Cincinnati, OH) liquid dishwashing detergent] was rubbed across the surface for 10 strokes with knitted polyester wipes (Quiltec I; Contec, Inc., Spartanburg, SC), and then the surface was rinsed with ASTM D5127-90 grade E-1 UPW. Ion concentrations of metals were in the low parts per trillion; total oxidizable carbon was about 2 ppb; and resistivity was greater than 18 MΩ. This water was produced by a continually flowing system (Hango *et al.*, 2000) in which 185-nm UV oxidizes organic carbon, ion exchange resins remove cations and anions, 254-nm UV kills microbes, and filters remove particles down to 0.04 μm. UPW produced in this way cannot stay clean if static and must be pulled off a continually flowing loop, which runs at 8 gpm and 60 psi.

This process was repeated three times with fresh washing solution. The coupons were continually submerged during this process because exposure to air causes detergent particulates to form. The final cleaning was accomplished in a 75°C UPW flowing bath, agitated by nitrogen, for 30 min. Coupons were then air-dried. The majority of the cleaning process was performed inside a Class 10 clean-room (Hango *et al.*, 2000). All Genesis mission (see http://genesismission.jpl.nasa.gov/) hardware were cleaned using this methodology.

5. *Semi-aqueous, multiple-solvent (SAMS) cleaning*. A commercially available fully automated system was used. Briefly, the system used acetic acid for removing microbes, followed by a deoxygenated metal cleaning solution to remove any remnants. Finally, coupons were rinsed with 18 MΩ, degassed, deionized water and dried with hot, clean nitrogen at 74–82°C. This cleaning procedure is used in semiconductor, medical, and other industries. The name of the manufacturer of this unit is not disclosed for commercial reasons.

Microbial examination for validating cleanliness

1. *Conventional microbial examination*. Some of the test coupons were placed into 5 ml of sterile water and sonicated for 2 min. After sonication, sample aliquots were then transferred into petri dishes, and cultivable spores were enumerated by pour plate technique using trypticase soy agar (TSA) as the growth medium (32°C for 3–7 days). Other test coupons were placed into trypticase soy broth (TSB) without sonication for qualitative analysis. The spores or their remains in the liquid solution were trapped onto a 0.2-μm (pore size) nitrocellulose filter. Both the filters and the metal coupon surfaces were stained with BacLight stain (Molecular Probes, Eugene, OR) to enumerate total number of spores. Suitable positive and negative controls were used for comparative analysis.
2. *Scanning electron microscopy*. A field-emission scanning electron microscope (FE-SEM) provides high spatial resolution combined with low electron beam accelerating voltage. The low-beam voltage of the FE-SEM allows examination of electrical insulators without having to deposit a surface-conducting (carbon or metal) layer to eliminate specimen charging, which can lead to a distorted and often completely unusable image. The deposition of a conducting material to control charging can complicate the analysis. In many situations, a low electron beam voltage intrinsically results in a much sharper image, especially of thin structures composed of elements of low atomic number. A Phillips (Hillsboro, OR) FE-SEM (XL-50) was used to analyze a majority of the samples. Elemental analysis is possible in a scanning electron microscope equipped with an energy-dispersive x-ray (EDX) analyzer. EDX is based on the analysis of the characteristic x-rays emitted when an electron beam is incident on a sample. Unfortunately, the spatial resolution obtainable with EDX is at best about 1 μm. Because of charging of the metal surface low acceleration voltage (~5 kV) was employed for the investigation of titanium samples. The acceleration voltage for analyzing aluminum samples was ~10–20 kV. In the high vacuum mode secondary electron images were acquired for both metals. Similar settings were maintained when different models or SEM instruments were used.
3. *Low-volatile residue (LVR) assay*. The coupons were sonicated for 2 min, and LVRs were extracted with dichloromethane. The LVRs were analyzed using diffuse reflectance/Fourier transform infrared (DRIFT/FTIR) spectroscopy. The analysis followed the ACL-120 procedure that complies with MIL-STD-1246C Notice 3 and is sensitive to the most stringent level (A/100). The DRIFT/FTIR spectroscopy provides chemical functional group information such as aliphatic hydrocarbon, silicon, phthalates and other esters, and amide groups for quantitative analysis and qualitative identification of materials.

RESULTS

Recovery of B. subtilis *spores from metal surfaces*

Table 1 shows spores recovered from both types of metal surfaces for each of the five cleaning methods studied. The commercially available spore vials manufactured by Difco Laboratories contained 1.5×10^9 spores/ml as evaluated by microscopy. About 53% of the spores present in these vials did not show any growth in TSA medium incubated at 32°C for 2–7 days. The ster-

TABLE 1. REMOVAL OF *B. SUBTILIS* SPORES FROM METAL SURFACES BY VARIOUS CLEANING PROCEDURES

	Number of cultivable spores (cfu/coupon) recovered after cleaning of metal			
	Aluminum (n = 12)		*Titanium* (n = 12)	
Cleaning method	*Minimum–maximum*	*Mean*	*Minimum–maximum*	*Mean*
Uncleaned[a]	3.2×10^2–3.6×10^3	1.5×10^3	2.6×10^2–4.8×10^3	3.0×10^3
IPA	0–8	8	1.8×10^2–3.7×10^2	1.1×10^2
Water	0–3.5×10^1	8	4.0×10^1–2.1×10^2	9.8×10^1
JPL	0–1.8×10^1	3	0[b]	0
JSC	0–2.0×10^1	4	3–3.3×10^1	1.9×10^1
SAMS	5.4×10^2–1.3×10^3	8.5×10^2	0[b]	0

cfu, colony-forming units.
[a]The recoverable spores from aluminum and titanium were 24% and 51%, respectively.
[b]The coupon placed in TSB did not show any growth.

ile Sigma water-washed spore suspension kept in water at 4°C for 1 year did not show any change in its total spore (microscopy) and cultivable spore counts (TSA growth). The metal coupons that were inoculated with a known amount of *B. subtilis* spores (1.4×10^4 total and 5.8×10^3 cultivable spores) and dried at room temperature without controlling the humidity (45% in Pasadena, CA) were recovered by sonication (2 min). As shown in Table 1, about 24% (1.5×10^3) and 51% (3.0×10^3) of cultivable spores were recovered from the inoculated aluminum and titanium coupons, respectively.

Cleaning efficiencies

Table 1 show the efficiency of various cleaning procedures used in this study for decontaminating *B. subtilis* spores from aluminum and titanium. Based on the cultivable spore counts on TSA, the SAMS method showed a 1-log reduction, and the other four cleaning methods showed a 3-log reduction, of *B. subtilis* spores from aluminum (Fig. 1). The mean recovery of the cultivable spores in the latter four methods was only 3–8 spores per aluminum coupon (Table 1).

In the case of titanium, two of the cleaning methods—the JPL and SAMS methods—cleaned titanium to "sterility," *i.e.*, spores were not cultured from the JPL- and SAMS-cleaned titanium coupons in TSA, and also prolonged incubation of coupons in TSB did not show any growth. This is a significant finding for life-detection science efforts. The recovery of spores from the titanium cleaned by the other three methods was high ($2–4 \times 10^2$ spores per coupon) compared with aluminum (4–8 spores per coupon).

In the case of aluminum, the removal of spores was difficult. The inoculation of the aluminum surface with spores was repeatedly coincident with the appearance of the formation of "dome-like" structures on the inoculated aluminum coupons (Fig. 2). The structures were absent in spore-free inoculation controls. In addition, the spore-inoculated and cleaned aluminum coupons released no cultivable bacteria when the coupons were not sonicated, regardless of the cleaning method used. However, once a coupon was sonicated, microbial colonies were easily released in solution and could be readily cultivated. As discussed below, it is feasible that the sonication process breaks open the "dome-like" structures and releases associated spores.

Although such "dome-like" structures were not observed on the titanium, sonication also im-

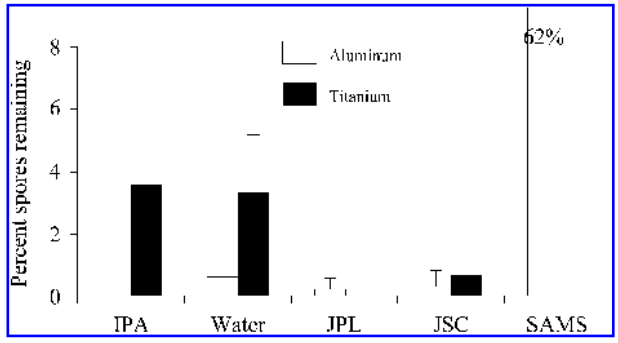

FIG. 1. Comparison of efficiencies of various cleaning methods in removing *B. subtilis* spores from both aluminum and titanium surfaces. The percentage of spores remaining is calculated by comparing the uncleaned metal coupons with the cleaned coupons after growth in TSA. The results are the average of six independent experiments. Bars represent the standard deviation.

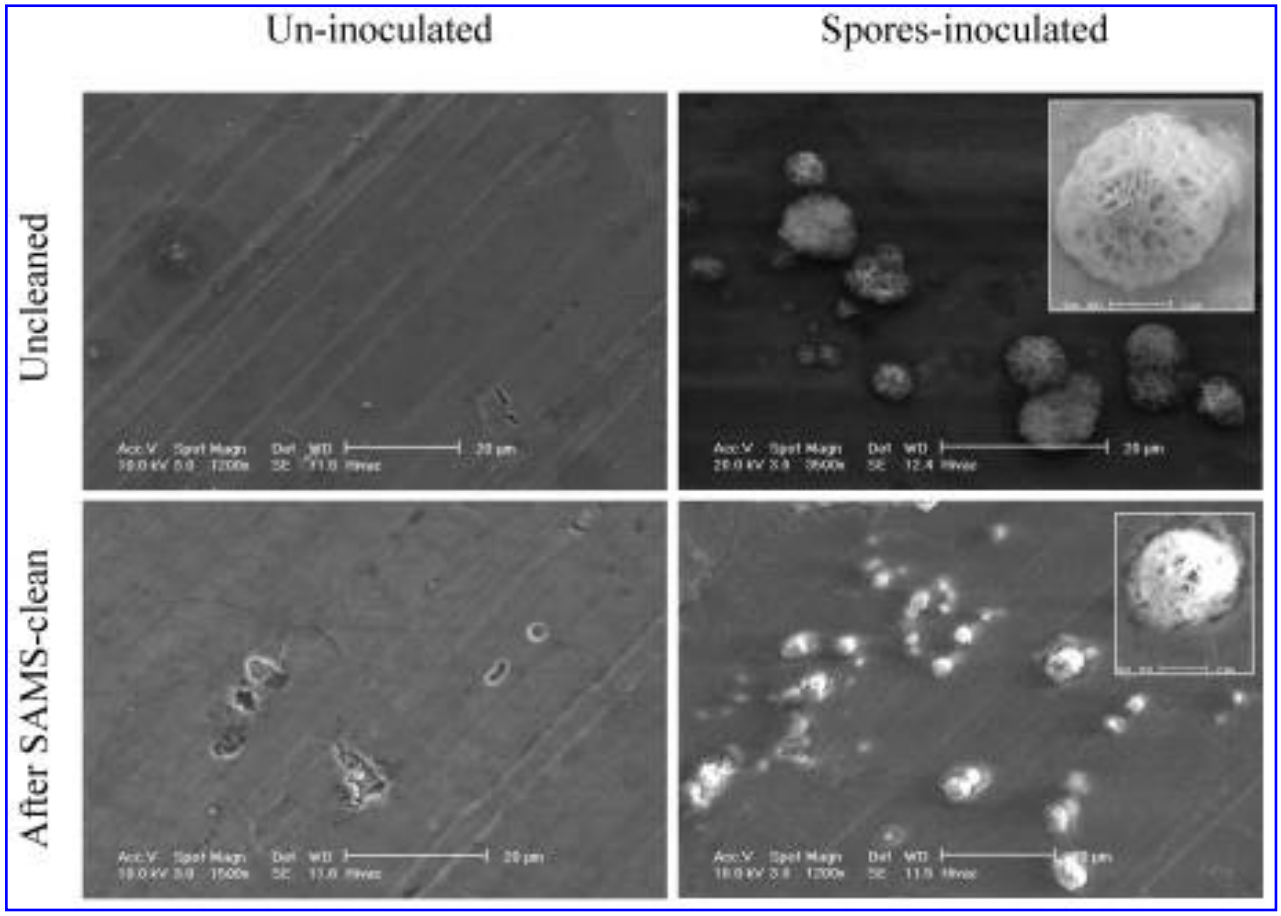

FIG. 2. "Dome-like" structure formation on *B. subtilis* spores inoculated on aluminum. *B. subtilis* spores were inoculated on precleaned aluminum coupons and dried at room temperature. When viewed under an FE-SEM, spore morphology was not seen. However, "dome-like" structures as shown here are clearly visible only on the aluminum coupons that were inoculated with spores. In addition, viable spores were cultured from the spore-inoculated aluminum after sonicated. **Insets, right panels:** Close-up images of the "dome-like" structures. Such a "dome-like" structure was not observed on titanium test coupons (data not shown).

proved the recovery of spores (data not shown). Furthermore, one of the cleaning methods, SAMS, contains surfactant in its cleaning solution that in fact served as a nutrient for the spores and allowed them to germinate (Fig. 3), particularly on the aluminum coupons. As Fig. 3 also shows, the vegetative cells (>4 μm) in these SAMS-cleaned aluminum coupons are clearly visible in epifluorescent microscopy, whereas the uncleaned aluminum that was inoculated showed only the presence of spores (<1 μm). Germination of spores was not seen in any other cleaning methods tested in this study.

The NASA standard procedure is to clean spacecraft materials by alcohol wiping (NASA, 1999). Spores display a recognizable morphology, which was used to identify them on titanium. In addition, spores on titanium undergo morphological changes after cleaning with alcohol wiping and water wiping, which is illustrated in Fig. 4. It is obvious from the results that alcohol wiping lysed open the spores and left behind remnants (Fig. 4B) on the titanium coupons. However, the cells were left intact after wiping with water (Fig. 4D). The spores inoculated on aluminum exhibited very interesting features after alcohol wiping and water wiping (see Fig. 5). As mentioned above, the *B. subtilis* spore suspension, after drying on the pits and flaws of the aluminum surface, appeared to be associated with "dome-like" structures. These structures were about 5–20 μm in size and were prevalent on all aluminum coupons that were inoculated with spores. Such structures were not observed when vegetative

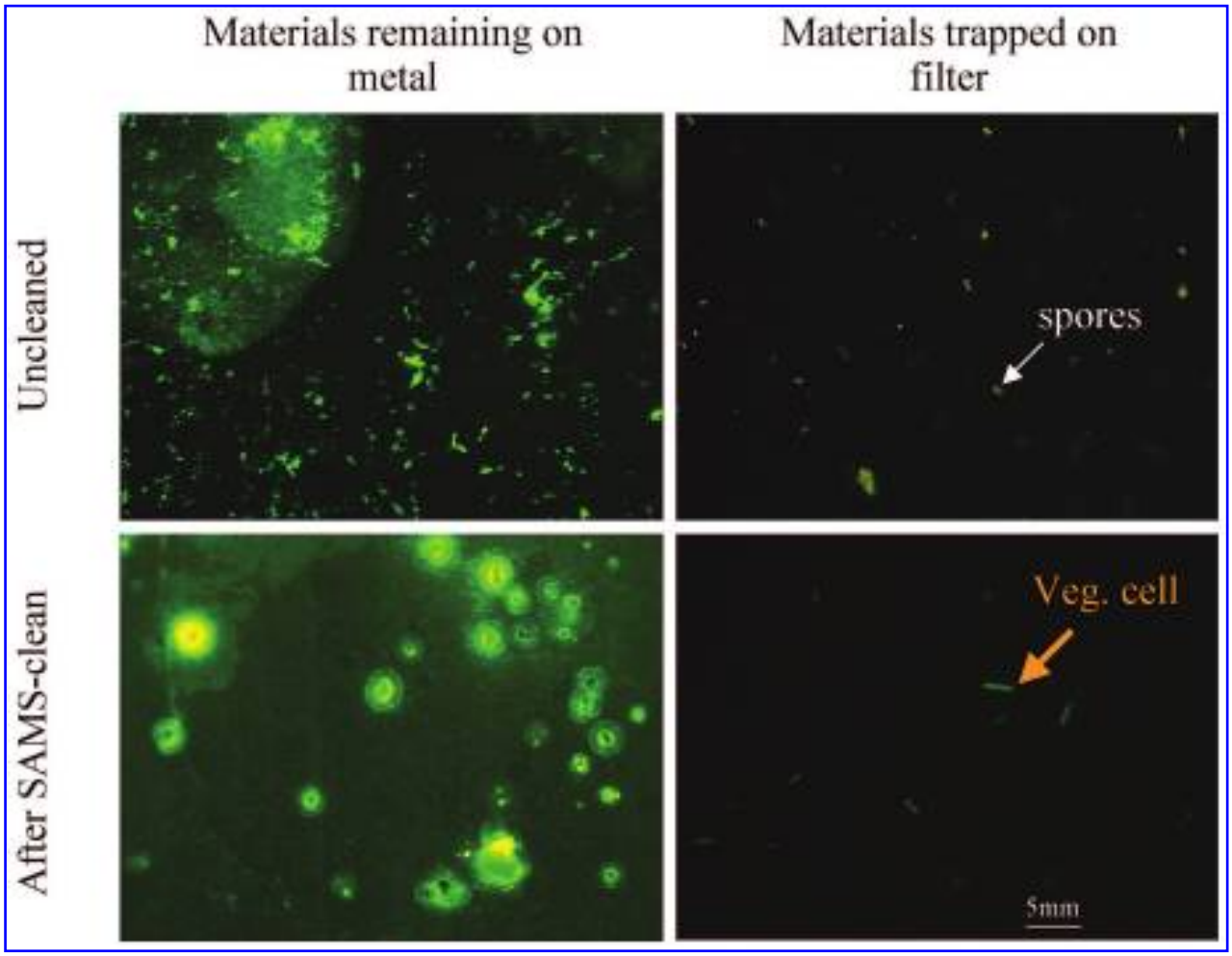

FIG. 3. Vegetative cell germination of *B. subtilis* spores after the SAMS cleaning method. Left panels: Epifluorescent micrographs of aluminum coupons inoculated with spores. **Right panels:** Materials removed from the metal coupons that were trapped onto a polycarbonate filter paper. Only spores were visible (white arrow; right upper panel) when the materials were trapped onto a polycarbonate membrane filter from the uncleaned aluminum coupons. After cleaning with the SAMS method, elongated vegetative cells were observed (brown arrow; right lower panel); presumably surfactants present in the cleaning solution promoted germination.

cells of Gram-positive and Gram-negative microorganisms were deposited on aluminum. Likewise, spores formed these structures to a lesser degree on various polished bare aluminum, and did not develop on chemically modified aluminum surfaces (data not shown).

The NASA-developed (JPL and JSC) cleaning methods decontaminated the spores effectively from aluminum and titanium, whereas the commercial system (SAMS) did not remove spores effectively from aluminum (Table 1). The biological remnants, assessed using a semi-automated system on an FE-SEM, are depicted in Figs. 5 (aluminum) and 6 (titanium). Bioremnants were not observed on aluminum; however, organic matter on titanium was identified by FE-SEM-EDX spectra. "Dome-like" structures were only sparse on the uninoculated aluminum coupons cleaned by the JPL method and were not observed in other cleaning methods. But, "dome-like" features were observed on all the inoculated aluminum coupons that were either cleaned by all five cleaning methods or left uncleaned.

DISCUSSION

Since very low levels of spores were spiked onto metal coupons (5.8×10^3 cultivable spores), a 2-log reduction by a cleaning method is diffi-

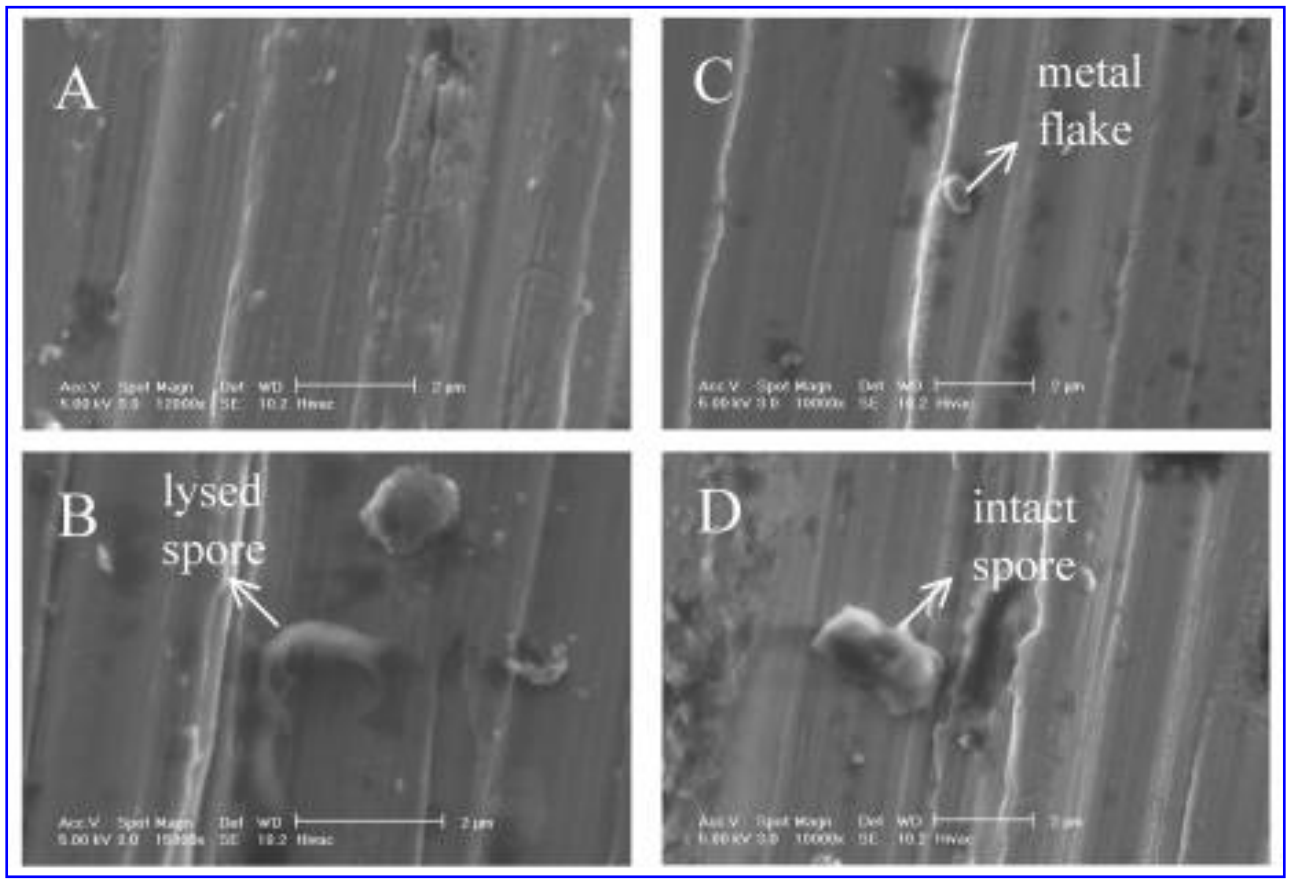

FIG. 4. Morphological changes of *B. subtilis* spores on titanium after cleaning with alcohol and water wiping. **A:** Precleaned and alcohol-wiped titanium metal coupon. **B:** *B. subtilis* spores are lysed open because of IPA wiping. **C:** A spore-like metal structure was observed in a precleaned and water-wiped titanium metal coupon. The EDX spectroscopy analysis revealed elemental composition of bare titanium, and the absence of carbon or any traces of calcium confirmed the structure is a "metal flake." **D:** Intact bacterial spore (EDX spectroscopy analysis revealed the presence of carbon and calcium) after water wiping.

cult to validate by conventional spore culture assay. Epifluorescent microscopy is a powerful tool to differentiate microbes (mostly vegetative cells and not spores) from particles after staining of the biological materials. However, both aluminum and titanium adsorb the stain used and emit a fluorescent background, making it difficult to validate the cleanliness of the materials after decontamination with microscopy. The removed biological materials can be trapped onto a filter (0.2 μm pore size; 13 mm in diameter) and observed under a microscope, but this filtration method requires a minimum of 10^5 cells to visualize any particle of spore size (1 μm) under oil immersion ($\times$2,000 magnification). Therefore, we used enzyme-based technologies to measure the spore bioburden; however, both ATP- (Venkateswaran *et al.*, 2002) and *Limulus* amebocyte lysate- (La Duc *et al.*, 2004) based assays do not measure low concentrations of spores. For this reason, a "plus or minus" sterility study was carried out by placing the whole coupon into TSB medium and incubating at appropriate cultural conditions. Similarly, DRIFT/FTIR measurements were inconclusive because the inoculated spore suspension was barely detected by this assay (50 ng per sample). However, DRIFT/FTIR measurements were beneficial for detecting nonspecific cross-contamination.

Selective adhesion of spores onto aluminum surfaces might be attributed to the surface oxidation due to sample preparation (autoclaving at 121°C for 15 min; 30-min drying). Ultimately this means that the aluminum actually remained more contaminated than we were able to verify using our sonication-filtration recovery techniques. Similarly,

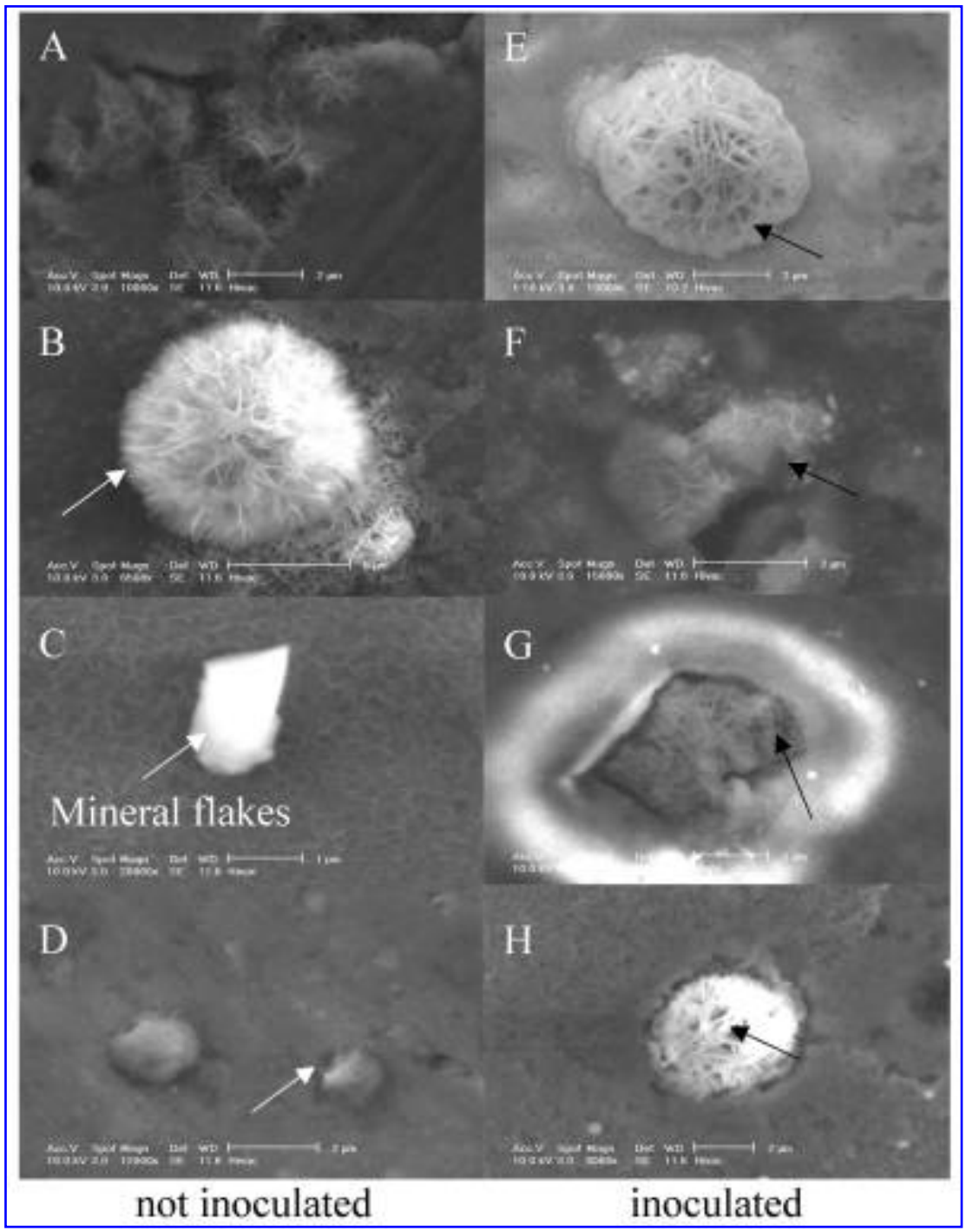

FIG. 5. Morphological features of spores on aluminum before and after various cleaning methods. A–D: FE-SEM micrographs of aluminum coupons not inoculated with spores. **E–H:** FE-SEM micrographs of aluminum coupons inoculated with spores that were cleaned by the various methods used in this study. A: Uninoculated and precleaned control. Aluminum showed pits and faults in several locations. B and F: JPL cleaning method. C and G: JSC cleaning method. D and H: SAMS cleaning method. E: Spore-inoculated uncleaned control. Spore morphology was not visible in any of the control aluminum or cleaned coupons. The "dome-like" structure is visible in images E–H (black arrows), and EDX spectroscopy analysis showed that these structures are rich in Mg and Ca. Similar structures were formed by the JPL cleaning method (B) as well as by the SAMS cleaning method (D) (white arrows), though the EDX spectrum did not reveal elemental composition such as Mg or Ca. In addition, bacterial growth was not observed in this set of samples after cleaning by the JPL and SAMS cleaning methods. The JSC-cleaned aluminum (C) showed some etching, and the EDX spectroscopy analysis revealed high concentration of Si in these structures.

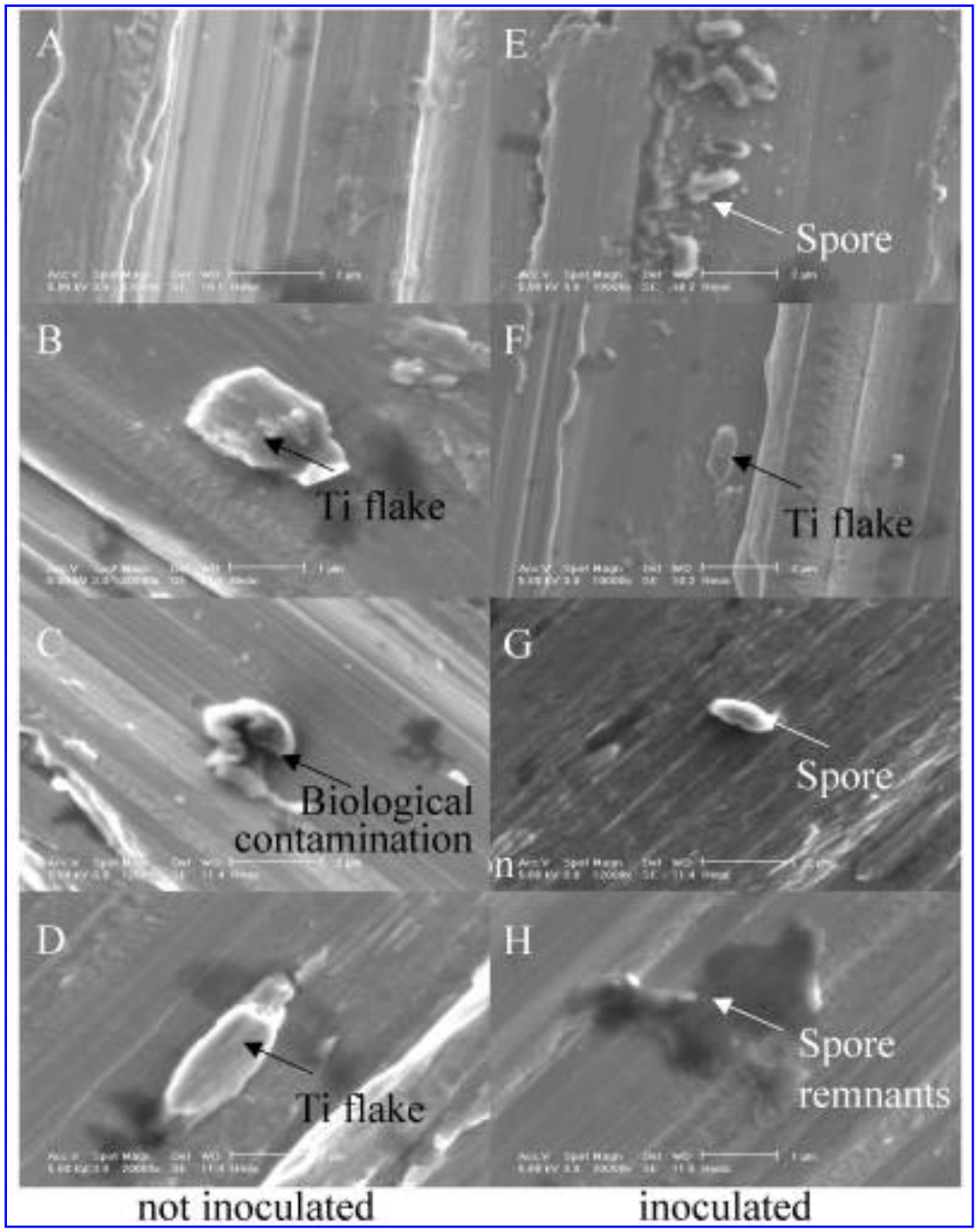

FIG. 6. Morphological features of spores on titanium before and after various cleaning methods. A–D: FE-SEM micrographs of titanium coupons not inoculated with spores. **E–H:** FE-SEM micrographs of titanium coupons inoculated with spores that were cleaned by various methods. A: Uninoculated and precleaned control. Titanium coupons showed ridges, but pits and faults, like those observed in aluminum, were not observed. B and F: JPL cleaning method. Spore-like structures were noticed in both spore-inoculated and uninoculated controls. These structures were confirmed as titanium flakes by EDX spectroscopy analysis because carbon or calcium, both elements characteristic to spores, was not detected even though elemental composition unique to titanium was documented. C and G: JSC cleaning method. Structures resembling biological matter were observed. The EDX spectra of these structures confirmed the presence of carbon as well as calcium in G. Subsequently, spores were isolated from JSC-cleaned titanium. Isolation of uninoculated microorganisms in C validated the secondary contamination during the JSC method cleaning process. D and H: SAMS cleaning method. As seen in B and F, the structure noticed in D exhibited elemental composition unique to titanium. Although spores were not isolated from SAMS-cleaned titanium, remnants of spores were observed. The EDX spectra revealed the presence of carbon and calcium in these structures.

higher recovery of spores from titanium metal might conceivably be ascribed to the non-adherence of spores onto titanium instead of the cleaning efficiency of various methods employed, *i.e.*, more spores were recoverable from the titanium via sonication-filtration than from the aluminum.

Among the NASA-developed cleaning methods, the JSC method appeared to carve out metal components of aluminum. These pits and mineral flakes were noticed in both inoculated and uninoculated aluminum coupons after cleaning (EDX spectroscopy identified these as SiO_2 crystals). While the yield strength, hardness, and surface roughness of the aluminum were not changed because of the JSC cleaning method, the non-ionic nature of the UPW used in the JSC method might be corrosive for aluminum but not titanium, which was not eroded. However, secondary microbial contamination other than inoculated spores was seen in the titanium cleaned by the JSC method. Although both the JPL and SAMS methods cleaned titanium to sterility, bioremnants were also seen in the SAMS-cleaned titanium coupons.

In the JPL method, the titanium cleaning procedure includes a nitric acid passivation step to oxidize the surfaces. This harsh step consists of a 30-min exposure to 5 *M* nitric acid at room temperature. We therefore tested the idea that it is the passivation step that sterilizes the titanium surface. In a follow-on trial, this passivation step was incorporated into the otherwise standard process to clean aluminum that was inoculated with spores. The effect of this passivation step in the removal of spores from both titanium and aluminum was tabulated (Table 2). It is clear from the results that all passivation treatments cleaned both aluminum and titanium to sterility. Furthermore, the "dome-like" structures were usually also removed from the aluminum metal due to the nitric acid passivation (Fig. 7). But, the passivation step also damaged the aluminum surface (Fig. 7), whereas this adverse effect was not observed in titanium (data not shown).

We hypothesize that the "dome-like" structures noticed on aluminum are the result of biomineralization loci that contain spores. It may be that the spores themselves initiated this process, or they may simply have become entrained in the structures because of the affinity for the dome's mineral composition. Further characterization of the "dome-like" structures observed on the aluminum coupons is being carried out and is of significant—although not crucial—importance to the findings of this study. Research to date has demonstrated that these structures are much more likely to develop on unpolished aluminum, and they tend to aggregate in eroded and uneven areas of the metal and grow in size over time, apparently thriving in a localized niche. It is unclear at this point whether these tens-of-microns-sized elevated structures are the intitiators of a biomineralization event or simply the beneficiaries of an inorganic process. Association of the "dome" structures with biological activity has not yet been demonstrated conclusively, but the data are suggestive of such a connection. The detection of iron-containing nodules beneath the "domes," as determined by Auger analysis, is perhaps the most compelling evidence seen to further this supposition (data not shown). Likewise, isolation of *B. subtilis* spores from the inoculated samples after sonication, which lysed open the structures, together with the absence of spore-like structures following subsequent electron microscopy analysis, suggest that spores may indeed reside beneath the "domes."

Table 2. Effect of Nitric Acid Passivation in the Removal of Spores from Various Metals

Metal	*Passivation*	*Number of trials tested*	*Number of trials showing growth*[a]
Aluminum (15 rms)[b]	Yes	12	0
	No	18	18
Titanium (32 rms)	Yes	18	0
	No	12	12

[a]The metal coupon immersed into TSB, and growth was checked after a 24-h incubation.
[b]The mirror-polished aluminum did not show any growth after passivation; however, six out of six coupons showed growth if the passivation step was not carried out.

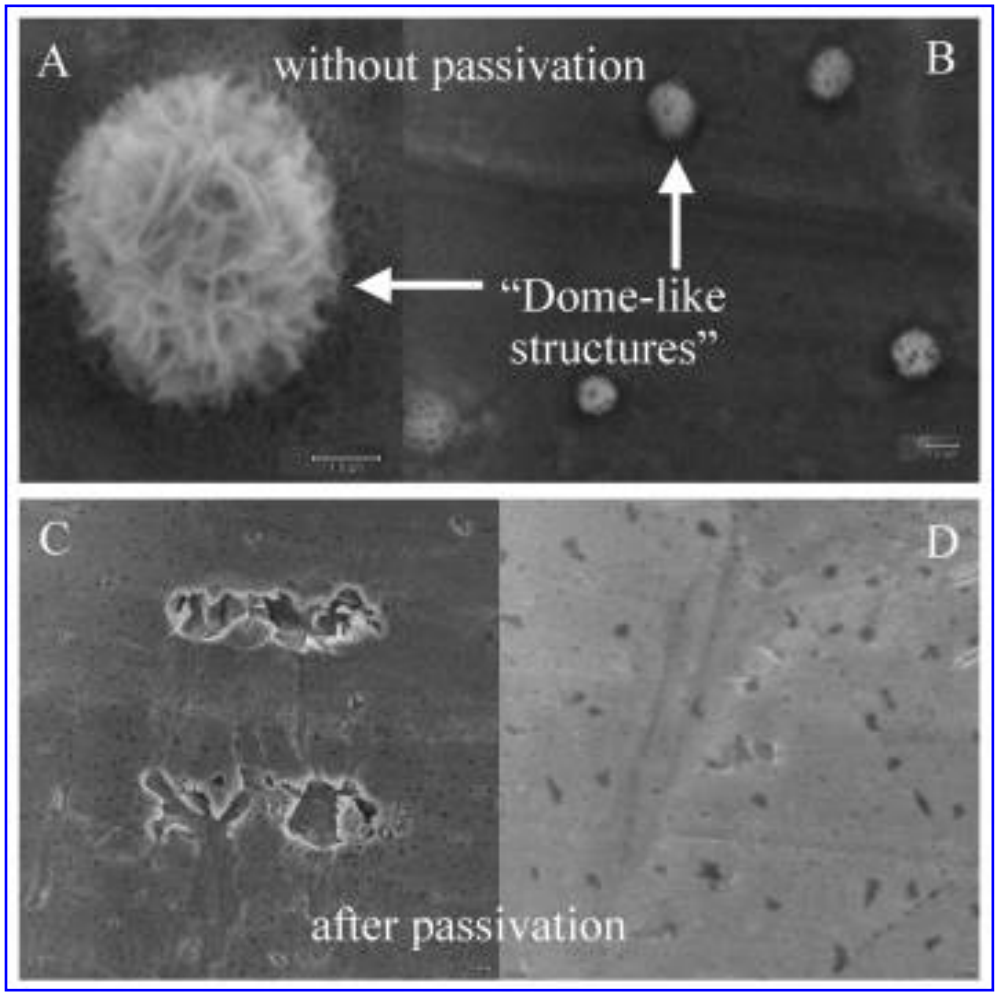

FIG. 7. Effect of nitric acid passivation on aluminum. All panels are aluminum metal coupons that were inoculated with spores and cleaned by the JPL cleaning method. As shown in Fig. 5 "dome-like" structures were observed (as shown in **A** and **B**) when the nitric acid passivation step was not carried out. However, when the nitric acid passivation step was performed (**C** and **D**), "dome-like" structures were removed, and aluminum metal was cleaned to sterility. However, major damage as well as etching to aluminum was noticed due to the nitric acid passivation step.

CONCLUSIONS

A study of the biological cleaning effectiveness of five cleaning methods and two materials commonly used in spacecraft assembly has shown that cleaning methods vary in their effectiveness, and that titanium is more conducive to cleaning to sterility than aluminum, which tends to degrade under the most effective cleaning regimes. The JPL multi-solvent cleaning method, documented in JPL Document FS505146C (Jet Propulsion Laboratory, 1990), seems to be the most effective cleaning method. For titanium, there was an additional nitric acid passivation step involved after alkaline cleaning. When this step was applied to aluminum on a trial basis it also cleaned to sterility but degraded the material's surface.

Results showed that all methods studied cleaned spores from the metal surfaces to some degree. However, the commercial SAMS cleaning process that is commonly used in the microchip industry is not recommended for biological sampling hardware. The treatment not only appears to lyse spores open, but also the surfactant acted as a culturing media for the *B. subtilis* spores used in the study, enabling the spores to germinate.

A significant finding of this study was that the pretreatment of the material by autoclaving degraded the aluminum, enabling the formation of

niches where spores adhered to the surface with apparently more tenacity and in greater numbers. While aluminum would not normally be pretreated by autoclaving prior to use on a spacecraft, we believe that autoclaving merely speeded up the normal oxidation process that occurs when aluminum is exposed to oxidizing, desiccative environmental conditions over a longer period of time. Spacecraft-borne sampling equipment can undergo several years of desiccative conditions in air before reaching the mission's destination, this makes aluminum surfaces a potential problem. Therefore, it is our conclusion that titanium (Ti 6Al-4V) should be considered superior to aluminum (Al 6061) for use in spacecraft sampling hardware, both for its potential to be cleaned to sterilization and for its hardiness to the most effective cleaning protocols. Furthermore, the compatibility of various spacecraft materials should be carefully considered when employing solvent-based and/or higher temperature-dependent commercial cleaning systems.

ACKNOWLEDGMENTS

We thank members of the Biotechnology and Planetary Protection group for technical assistance and discussion and K. Buxbaum and T. Luchik for valuable advice and encouragement. We acknowledge C. Allen, J. Edens, P. Koen, and J. Kulleck for assistance performing the electron microscopy.

ABBREVIATIONS

EDX, energy-dispersive x-ray; DRIFT/FTIR, diffuse reflectance/Fourier transform infrared; FE-SEM, field-emission scanning electron microscope; IPA, isopropyl alcohol; JPL, Jet Propulsion Laboratory; JSC, Johnson Space Center; LVR, low-volatile residue; SAMS, semi-aqueous, multiple-solvent; TSA, trypticase soy agar; TSB, trypticase soy broth; UPW, ultrapure water.

REFERENCES

Diaz, R.B., Vessoni Penna, T.C., Takeuti, C.E., Taira, E.T., and Guimarães, T. (2000) Centralization of disinfection of semi-critical and non-critical items in a Brazilian Hospital (Saint Cruz Hospital): Economical improvement of sanitizing procedures. *Laes Haes* 128, 68–78.

Faille, C., Jullien, C., Fontaine, F., Bellon-Fontaine, M.N., Slomianny, C., and Benezech, T. (2002) Adhesion of *Bacillus* spores and *Escherichia coli* cells to inert surfaces: Role of surface hydrophobicity. *Can. J. Microbiol*. 48, 728–738.

Hamon, M.A. and Lazazzera, B.A. (2001) The sporulation transcription factor Spo0A is required for biofilm development in *Bacillus subtilis*. *Mol. Microbiol*. 42, 1199–1209.

Hango, R.A., Warren, J., Allton, J., and Stansbery, E. (2000) High purity water used for Moon and Mars specimen curation. *Ultrapure Water* 17, 75–78.

Jet Propulsion Laboratory (1990) *Detail Specification, General Cleaning of Materials*, JPL Internal Document FS505146C, California Institute of Technology, Pasadena.

La Duc, M.T., Nicholson, W.L., Kern, R., and Venkateswaran, K. (2003) Microbial characterization of the Mars Odyssey spacecraft and its encapsulation facility. *Environ. Microbiol*. 5, 977–985.

La Duc, M.T., Kern, R., and Venkateswaran, K. (2004) Microbial monitoring of spacecraft associated environments. *Microb. Ecol.* 47, 150–158.

Link, L., Sawyer, J., Venkateswaran, K., and Nicholson, W. (2004) Extreme spore UV resistance of *Bacillus pumilus* isolates obtained from an ultraclean spacecraft assembly facility. *Microb. Ecol.* 47, 159–163.

McDonnell, G. and Russell, A.D. (1998) Antiseptics and disinfectants: Activity, action, and resistance. *Clin. Microbiol. Rev*. 12, 147–179.

Military Standard 1246C (1998) *Product Cleanliness Levels and Contamination Control Program, June 15, 1998 (now IEST-STD-CC1246D, Feb. 15, 2002)*, California Institute of Technology, Pasadena.

NASA (1999) *Biological Contamination Control for Outbound and Inbound Planetary Spacecraft, Policy Directive NPD 8020.7^{E}*, NASA, Washington, DC.

Penna, T.C., Mazzola, P.G., and Silva Martins, A.M. (2001) The efficacy of chemical agents in cleaning and disinfection programs. *BMC Infect. Dis*. 1, 16–24.

Rutala, W.A. and Weber, D.J. (1998) Principles of disinfecting patient-care items. In *Disinfection, Sterilization and Antiseptics in Health Care*, edited by W.A. Rutala, Polyscience Publishers, New York, pp. 133–149.

Sagripanti, J.L. and Bonifacino, A. (1997) Effects of salt and serum on the sporocidal activity of liquid disinfectants. *J. AOAC Intern*. 80, 1198–1207.

Space Study Board (1992) *Biological Contamination of Mars: Issues and Recommendations*, Commission on Physical Sciences, Mathematics and Applications, National Research Council, Washington, DC.

Venkateswaran, K., Satomi, M., Chung, S., Kern, R., Koukol, R., Basic, C., and White, D. (2001) Molecular microbial diversity of a spacecraft assembly facility. *Syst. Appl. Microbiol*. 24, 311–320.

Venkateswaran, K., Hattori, N., La Duc, M.T., and Kern, R. (2002) ATP as a biomarker for viable microorgan-

isms in clean-room facilities. *J. Microbiol. Methods* 52, 367–377.

Venkateswaran, K., Kempf, M., Chen, F., Satomi, M., Nicholson, W., and Kern, R. (2003) *Bacillus nealsonii* sp. nov., isolated from a spacecraft assembly facility, whose spores are gamma-radiation resistant. *Int. J. Syst. Evol. Microbiol*. 53, 165–172.

Weber, D.J. and Rutala, W.A. (1998) Occupational risks associated with the use of selected disinfectants and sterilants. In *Disinfection, Sterilization and Antiseptics in Health Care*, edited by W.A. Rutala, Polyscience Publishers, New York, pp. 217–220.

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1. Christine Moissl, Shariff Osman, Myron T. La Duc, Anne Dekas, Eoin Brodie, Tadd DeSantis, Kasthuri Venkateswaran. 2007. Molecular bacterial community analysis of clean rooms where spacecraft are assembled. *FEMS Microbiology Ecology* **61**:3, 509. [CrossRef]